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Fks1 and Fks2 Are Functionally Redundant but Differentially Regulated in *Candida glabrata*: Implications for Echinocandin Resistance

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The echinocandins caspofungin, micafungin, and anidulafungin, inhibitors of cell wall β-1,3-glucan synthesis, were recently elevated to first-line agents for treating infections due to the azole-refractory yeast *Candida glabrata*. In *Candida albicans*, echinocandin resistance is strictly associated with mutations in Fks1, a large integral membrane protein and putative β-1,3-glucan synthase, while mutations in both Fks1 and its paralog Fks2 (but not Fks3) have been associated with resistance in *C. glabrata*. To further explore their function, regulation, and role in resistance, *C. glabrata fks* genes were disrupted and subjected to mutational analysis, and their differential regulation was explored. An *fks1*Δ *fks2*Δ double disruptant was not able to be generated; otherwise, all three single and remaining two double disruptants displayed normal growth and echinocandin susceptibility, indicating Fks1-Fks2 redundancy. Selection on echinocandin-containing medium for resistant mutants was dependent on strain background: only *fks1*Δ and *fks1*Δ *fks3*Δ strains consistently yielded mutants exhibiting high-level resistance, all with Fks2 hot spot 1 mutations. Thus, Fks1-Fks2 redundancy attenuates the rate of resistance; further analysis showed that it also attenuates the impact of resistance-conferring mutations. Growth of the *fks1*Δ and, especially, *fks1*Δ *fks3*Δ strains was specifically susceptible to the calcineurin inhibitor FK506. Relatedly, FK506 addition or calcineurin gene *CMP2* disruption specifically reversed Fks2-mediated resistance of laboratory mutants and clinical isolates. RNA analysis suggests that transcriptional control is not the sole mechanism by which calcineurin modulates Fks2 activity.

In recent decades, the normally commensal yeast *Candida glabrata* has emerged as a common cause of life-threatening fungal infection, in large part a consequence of its intrinsically low susceptibility or resistance to widely used azole antifungals. The echinocandins caspofungin (CSF; FDA approved in 2001), micafungin (MCF; 2005), and anidulafungin (ANF; 2006) are lipopeptide inhibitors of β-1,3-glucan synthase and, hence, fungal cell wall synthesis (6, 8, 35). They are highly active versus most *Candida* species, including *C. glabrata* (32, 37). Consequently, recent guidelines have elevated echinocandins above azoles as first-line agents for treatment of *C. glabrata* infection (33).

Echinocandin resistance remains a rare event but appears to be increasing in response to increasing clinical use (38, 39, 40, 45). Resistance was initially described in clinical isolates of *Candida albicans* (8, 34) and associated with mutations in the essential gene *FKS1* (also known as *GSC1*), encoding a large integral membrane protein and the putative β-1,3-glucan synthase (10, 29). To date, 18 distinct mutations in Fks1 of *C. albicans* or related species, *Candida tropicalis* and *Candida krusei*, have been reported, and all but 2 fall within an 8-residue region known as hot spot 1 (Fig. 1). In the genetic model *Saccharomyces cerevisiae*, resistance-conferring mutations selected *in vitro* are similarly limited to Fks1 except in *fks1*Δ disruptants in which comparable mutations occur in the paralog Fks2 (8, 21, 31, 34). Thus, in *S. cerevisiae*, Fks1 and Fks2 are functionally redundant, albeit only partially since *fks1*Δ strains grow slowly (19, 27). Furthermore, their expression in *S. cerevisiae* is differentially regulated: *fks1* is constitutive, while *fks2* is minimally expressed unless induced by glucose deprivation, pheromone, or calcium in a calcineurin-dependent manner (27). Indeed, the calcineurin inhibitor FK506 sensitivity is the basis for the gene name *FKS1* (9). In wild-type (WT), mating-proficient *S. cerevisiae*, Fks1 is responsible for cell wall synthesis during vegetative growth, while Fks2 and a third paralog, Fks3, share this responsibility during sporulation (19, 20, 27).

C. glabrata is an evolutionarily close relative of *S. cerevisiae* (41), and its genome includes syntenic orthologs of *FKS1*, *FKS2*, and *FKS3* (24). However, mating and sporulation have never been observed in this haploid yeast, and hence, the respective roles and regulation of these *FKS* genes are a matter of some interest. An intriguing clue has been provided by analysis of echinocandin resistance mutations in *C. glabrata* clinical isolates: in contrast to other *Candida* species and *S. cerevisiae*, these mutations involve both Fks1 and Fks2 (Fig. 1). Indeed, Fks2 mutations outnumber Fks1 mutations 2 to 1 in this yeast (50 versus 24, respectively) (3, 4, 5, 13, 16, 24, 28, 39, 40, 45). Furthermore, *fks1* and *fks2* are expressed at comparable levels in most strains examined (16).

Here we employed gene disruption, *in vitro* selection of resistant mutants, FK506 treatment, and expression analysis to explore the roles of *fks1*, *fks2*, and *fks3* in *C. glabrata* growth and echino-

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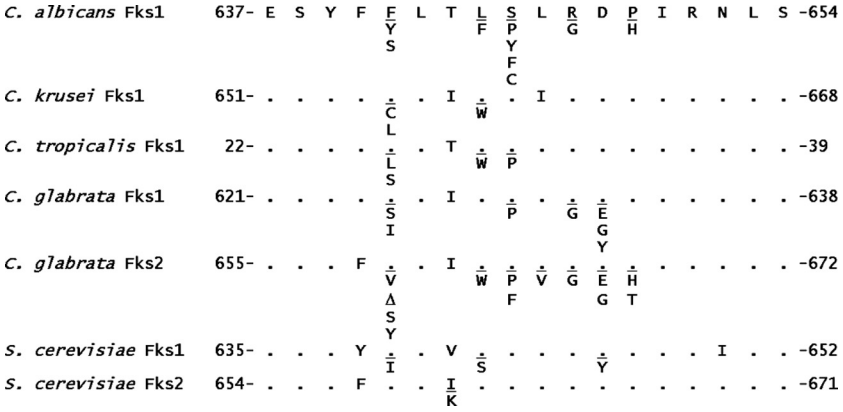

FIG 1 Fks1 and Fks2 hot spot 1 mutations associated with echinocandin resistance in clinical isolates of *C. albicans*, *C. krusei*, *C. tropicalis* (partial sequence), and *C. glabrata* and laboratory mutants of *S. cerevisiae* (2, 3, 4, 5, 7, 15, 16, 22, 24, 26, 28, 30, 31, 34, 39, 40, 45). Dots indicate identity at that position to the *C. albicans* sequence. Mutations are shown beneath the underlined wild-type residue. Δ, deletion.

candin susceptibility. Consistent with clinical data and in contrast to other yeasts, we observed that *fks1* and *fks2* are functionally fully redundant, with the latter a preferred target for resistance mutation. We also demonstrate how their differential regulation can be exploited to reverse Fks2-mediated resistance.

MATERIALS AND METHODS

Strains, media, and drugs. *C. glabrata* strain 200989 (*ura3 trp1*Δ *his3*Δ) (25) was obtained from the American Type Culture Collection (Manassas, VA); the clinical isolates 20409 (24) and DPL23 to DPL41 (16) were previously described. The medium was 1% yeast extract, 2% peptone, and 2% dextrose (YPD) or, where indicated, synthetic defined (SD)-ura or SD-trp (dropout base [DOB] supplemented with complete supplement mixture [CSM]-ura or CSM-trp; Sunrise Science Products, San Diego, CA). ANF (Eraxis) was obtained from Pfizer (New Yori, NY), CSF (Cancidas) was obtained from Merck (Rahway, NJ), MCF (Mycamine) was obtained from Astellas (Deerfield, IL), terbinafine (Lamisil) was obtained from Novartis (East Hanover, NJ), and FK506 was obtained from Tecoland (Edison, NJ). Drug stocks were prepared in 100% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO) and stored at −20°C. DNA primers (Table 1) were obtained from IDT (Coralville, IA).

Construction of *fks*Δ and *cmp2*Δ disruptants. The PRODIGE method was used to disrupt the *C. glabrata* orthologs of *FKS1* and *FKS2* (GenBank accession numbers XM_446406 and XM_448401, respectively) in strain 200989 essentially as described previously (12). Briefly, plasmid pRS416 (GenBank accession number U03450) was used as the template with *Taq* polymerase (New England BioLabs, Ipswich, MA) to amplify disruption cassettes consisting of the *URA3* coding region fused to sequences immediately upstream of the *FKS1* or *FKS2* start codon (primer CgFKS1c1-URA3F or CgFKS2c1-URA3F, respectively) and sequences immediately downstream of *FKS1* codon 635 or the *FKS2* stop codon (CgFKS1c635-URA3R or CgFKS2c1898-URA3R, respectively). A *cmp2*Δ (GenBank accession number XM_449251) disruption cassette was similarly amplified (primers CgCMP2c1-URA3F and CgCMP2c576-URA3R). Since *FKS3* is minimally expressed (16), in lieu of the PRODIGE method, a conventional disruption cassette was amplified with a plasmid pRS414 template (GenBank accession number U03448) which fused *FKS3* upstream (primer CgFKS3c1-TRP1F) and downstream (CgFKS3c1841-TRP1R) sequences to *TRP1* coding plus flanking sequences. Following transformation of these PCR products into strain 200989 with selection on SD-ura or SD-trp, colonies were streaked for isolation and screened by PCR with appropriate primer pairs (Table 1) and sequencing (see below).

Susceptibility assays. MIC (≥80% inhibition relative to the drug-free control) was determined by broth microdilution in YPD medium with incubation at 35°C for 24 h as described previously (44). Where indicated, FK506 was added to cultures prior to aliquoting to the 96-well plates. Selected assays were repeated in RPMI 1640 (Sigma-Aldrich) supplemented with 2% dextrose, 0.165 M MOPS (morpholinepropanesulfonic acid) (pH 7), and CSM (Sunrise Science Products).

Selection and characterization of resistant mutants. Mutant colonies were selected on echinocandin-containing agar medium overlaid with 1×10^8 cells of the indicated strain, followed by incubation at 35°C for 3 to 4 days, and the three largest colonies were streaked for isolation on drug-free plates. Susceptibility assays were used to identify stably resistant mutants, and the two exhibiting the highest MICs were further analyzed. Hotspot 1 regions of *FKS1* and *FKS2* were subsequently amplified (24), and PCR products were directly sequenced (Genewiz, South Plainfield, NJ) following treatment with exonuclease I plus thermosensitive alkaline phosphatase (New England BioLabs).

Mutants of the *C. glabrata* ortholog (GenBank accession number XM_448641) of *S. cerevisiae FPR1* were selected on medium containing the synergistic combination of 16 μg/ml terbinafine plus 0.3 μg/ml FK506, followed by screening for unaltered terbinafine susceptibility; amplification and sequencing (Table 1) of two mutants identified an F106L substitution and a single base deletion within codon K78.

Expression analysis. RNA was prepared from log-phase cultures (treated as indicated) using hot SDS-phenol extraction as previously described (44). *FKS* expression was assessed using one-step SYBR green quantitative reverse transcription-PCR (qRT-PCR) with the Mx3005P multiplex quantitative PCR system (Stratagene, La Jolla, CA), as previously described (14). Primers are listed in Table 1. Assays were performed in triplicate, and expression ratios were calculated using the Pfaffl method (36).

RESULTS

Disruption of *C. glabrata* Fks genes minimally affects growth and echinocandin susceptibility. For these studies, we employed *C. glabrata* strain 200989 (25), a *ura3 trp1*Δ *his3*Δ derivative of CBS138 (ATCC 2001), the strain selected for genome sequencing (11). This auxotrophic strain grows well in standard YPD medium but not in RPMI, the standard for susceptibility assays in the clinical laboratory. Hence, YPD was routinely employed; representative susceptibility assays repeated in supplemented RPMI medium generated qualitatively similar results (not shown). Disruptants were generated with a *ura3* or *trp1* marker or a combination of the two. Compared to their 200989 parent, the single disruptants *fks1*Δ, *fks2*Δ, and *fks3*Δ had no apparent effect on growth; this was

TABLE 1 DNA primers used in this study

Application and primer[a]	Sequence (5′–3′)[b]
Gene disruption	
CgFKS1c1-URA3F	TCTTTCATTTCTAGAGTTTTATCTTTTTTTTTTGCTCTTGT TTGATATACATTCGCTATGTCGAAAGCTACATATAAGGA
CgFKS1c635-URA3R	AGTGGTAGACAAAATTCTGATTGGATCTCTTAGAGATAGA ATCAAGAAGTAGTATGATTCGGTATTTCACACCGCATAGG
CgFKS2c1-URA3F	CAAGTCTCTATCAGCCAATAAAGGAATAAGAACAAGACAG AAAAAGAAAATTCCAACCATGTCGAAAGCTACATATAAGG
CgFKS2c1898-URA3R	ATTCCTAATTAGAAAAATTCTTGAAAATCATACTCAATTA GGGGATTATCTATTGCCTCTTTAGTTTTGCTGGCCGCATC
CgFKS3c1-TRP1F	AGTTTAGATATTCGTGAGACCAGTGAGGCCGCAGATTTAA TTATATTGGAATTAGGCTAGAGAGTGCACCATAAACGAC
CgFKS3c1841-TRP1R	TTATTGAGTTTCGGTGATTTCAATTCTTCAACTTTAAACG GATCCATCACTCATTACTATTTCTTAGCATTTTTGACGAA
CgCMP2c1-URA3F	TTGAATATATATTCTTGTTGGCACCGACAATATTTTCTAG TGCTTATAAATAAAAGATGTCGAAAGCTACATATAAGG
CgCMP2c576-URA3R	TAAATACGATATCAATATTATTATTTTGCTCTTGAGATGA AAAATAATTCGTGAGTAGGATTAGTTTTGCTGGCCGCATC
PCR screening and sequencing	
CgFKS1u198F	TCCCTATCGCTTAGGAAAAGT
CgFKS1c558F	GTTGCAGTCGCTACATTGCTA
CgFKS1c743R	TAGCGTTCCAGACTTGGGAA
CgFKS2u303F	AAGGACATCATACAGCGGTA
CgFKS2c594F	GGCCACTGTTTTATTCTTCTCG
CgFKS2c787R	GTAAATGTTCTCTGTACATGGA
CgFKS3u97F	ACTCTAATAACCTTGGAACTAG
CgCMP2u190F	CAGAGGATATCCCTTACCCA
CgCMP2c443R	TCAGGATCTCCACCAATCTC
CgFPR1u324F	AGCCCAAAATACGCCATTAAC
CgFPR1d84R	GTGATAAGGTTGTAAAGCGCA
ScURA3c117R	CTGCCCATTCTGCTATTCTG
ScTRP1c115R	TGGCAAACCGAGGAACTCTT
qRT-PCR	
CgFKS1c67F	TACCAACCAGAAGACCAACAGAATGG
CgFKS1c146R	TCACCACCGCTGATGTTTGGGT
CgFKS2c27F	CAATTGGCAGAACACCGATCCCAA
CgFKS2c172R	AGTTGGGTTGTCCGTACTCATCGT
CgFKS3c505F	GGGAGAGCACGTAAACGTAACTCAA
CgFKS3c671R	TTTGCTGCTGTAAGGTTAGTGGCG
CgURA3c210F	CGAGAACACTGTTAAGCCATTG
CgURA3c370R	CACCATGAGCGTTGGTGATA

[a] c, coding sequence; u, upstream (the number represents the nucleotide position of the 5′ end relative to the start codon); d, downstream (the number represents the nucleotide position of the 5′ end relative to the stop codon).
[b] Underlined nucleotides indicate *URA3* or *TRP1* sequences from pRS416 or pRS414, respectively.

also true for the double disruptants *fks1*Δ *fks3*Δ and *fks2*Δ *fks3*Δ (data not shown). These *C. glabrata* results are similar to what has been described for *S. cerevisiae*, with the exception that *fks1*Δ disruptants of the latter yeast show a partial growth defect. In *S. cerevisiae*, *fks1*Δ *fks2*Δ double disruptants are synthetically lethal, indicating that these genes are redundant for an essential function (27). Similarly, repeated attempts to generate a *C. glabrata fks1*Δ *fks2*Δ double disruptant with *trp1* and *ura3* markers failed (data not shown).

The parent strain 200989 exhibited MICs for ANF, CSF, and MCF of 0.016, 0.06, and 0.016 μg/ml, respectively. None of the single or double disruptants exhibited a significant (>2-fold) change in susceptibility to any of the three echinocandins. This contrasts with the 16-fold increase in CSF susceptibility of an *S. cerevisiae fks1*Δ strain (21, 23).

Rates and impacts of resistance-conferring mutations are altered in *fks1*Δ strains. Although *fks* disruption did not alter echinocandin susceptibility, we hypothesized that the loss of Fks1-Fks2 redundancy would alter the rate and magnitude of echinocandin resistance. To test this, 1×10^8 cells of each strain were spread on YPD plates containing echinocandin at concentrations corresponding to 16× the MIC (0.25, 1, and 0.25 μg/ml concentrations of ANF, CSF, and MCF, respectively). After 3 to 4 days incubation, it was apparent that there was considerable variation in the number and size of mutant colonies recovered (data not shown). The three largest colonies from each plate were streaked for isolation on drug-free YPD and then screened for resistance to the selection drug. ANF, CSF, and MCF susceptibilities were then determined for the two most-resistant mutants from each of the 6 strains and 3 selections. The results are summarized (Fig. 2) as fold changes in ANF, CSF, and MCF MICs relative to that of the wild-type parent. There was a marked difference between strains, as only *fks1*Δ strains (*fks1*Δ and *fks1*Δ *fks3*Δ) consistently yielded mutants exhibiting substantially increased MICs. Specifically, of the 12 *fks1*Δ mutants (i.e., 2 mutants for each of the 3 echinocandins for each of the 2 *fks1*Δ strains), 11 exhibited MIC increases of ≥64-fold. In contrast, only 2 of 12 mutants of *fks2*Δ strains and 0 of 12 mutants of wild-type and *fks3*Δ strains exhibited MIC increases of ≥64-fold. The difference in resistance rate and magnitude between *fks1*Δ strains and the wild-type or *fks3*Δ strains can be explained by Fks1-Fks2 redundancy and will be more directly examined below. Aspects of these data that elude explanation are the higher rate and magnitude of resistance in *fks1*Δ strains than in *fks2*Δ strains (see Discussion) and the failure to recover ANF-resistant mutants of the *fks1*Δ *fks3*Δ strain.

Resistance-conferring mutations selected *in vitro* are similar to those reported in clinical isolates. Genomic DNAs were prepared from 19 resistant mutants (selected to be representative but also to address the effects of redundancy), and their Fks1 or Fks2 mutations were identified by sequence analysis of PCR products. Sequencing results and MICs are summarized in Table 2. All 19 mutants exhibited a substitution or deletion within hot spot 1, consistent with previous analyses of resistant clinical isolates (Fig. 1). The Fks2 mutations F659Δ (11 mutants) and S663F (3 mutants) were repeatedly identified; these, along with Fks1-S629P (1 mutant), were previously reported in clinical isolates (7, 16, 45). The Fks1 mutations F625Δ (2 mutants), F625C, and P633T were novel, although clinical isolates with equivalent mutations in *C. glabrata* Fks2 or *C. krusei* Fks1 were previously reported (Fig. 1).

Fks1-Fks2 redundancy attenuates the impact of resistance-conferring mutations. Three pairs of mutants presented in Table

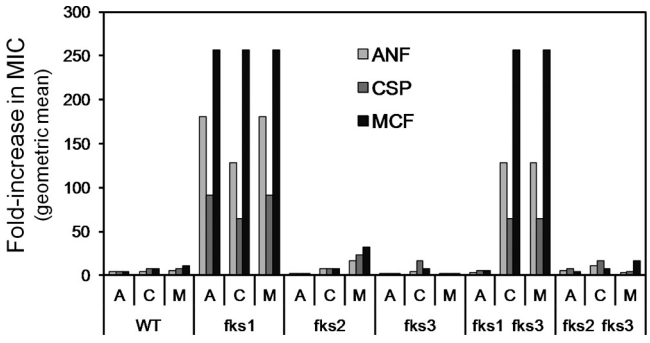

FIG 2 Differential effects of *C. glabrata* strain background on echinocandin resistance of laboratory-selected mutants. For each of the 6 strains and 3 selections, the 2 most-resistant mutants derived from the 3 largest colonies were assayed by broth microdilution for susceptibility to ANF (A), CSF (C), and MCF (M). The fold increase in MIC (relative to the wild-type parent of that strain), represented as the geometric mean for those 2 mutants, is shown.

TABLE 2 Echinocandin susceptibilities of *C. glabrata* wild-type and *fks*Δ strains and their echinocandin-selected mutants

Strain	Mutant	Mutation[a]		MIC (μg/ml)		
		Fks1	Fks2	ANF	CSF	MCF
WT parent		WT	WT	0.016	0.06	0.016
	A1, C2, M2	WT	F659Δ	0.03	0.12	0.06
	A2	WT	S663F	0.12	0.25	0.06
	C1	F625Δ	WT	0.12	2.0	0.25
	M1	S629P	WT	0.25	2.0	0.5
*fks1*Δ			WT	0.016	0.03	0.008
	A1, C1, C2, M2		F659Δ	2	2	2
	A2, M1		S663F	4	4	2
*fks2*Δ		WT		0.016	0.03	0.008
	C1	F625C		0.5	1	0.25
	M1	F625Δ		2	>16	4
*fks1*Δ *fks3*Δ			WT	0.016	0.03	0.008
	C1, C2, M1, M2		F659Δ	2	2	2
*fks2*Δ *fks3*Δ		WT		0.016	0.03	0.008
	A1	P633T		0.25	1	0.12

[a] Sequencing was restricted to the DNA encoding Fks1 residues 570 to 736 or Fks2 residues 608 to 779. WT, wild type.

TABLE 3 Reversal of Fks2-mediated echinocandin resistance by FK506

Strain[a]	Fks1	Fks2	Fold decrease in MIC due to FK506[b]		
			ANF	CSF	MCF
200989	WT[c]	WT	2	2	1
200989-C1	F625Δ	WT	1	1	1
200989-M1	S629P	WT	0.5	1	1
DPL38	F625S	WT	2	1	1
DPL39	S629P	WT	2	4	1
DPL41	D632G	WT	1	2	1
200989-A1	WT	F659Δ	4	8	8
200989-A2	WT	S663F	8	8	8
DPL23	WT	F659Δ	8	128	64
DPL34	WT	P667T	4	4	8
20409	WT	F659V	4	8	4

[a] See Table 2 for MICs of strain 200989 and its laboratory-derived mutants. The DPL (16) and 20409 (24) clinical isolates were previously described, and their MICs were reassayed for this study.
[b] Echinocandin MICs were determined in the absence or presence of FK506 (0.3 μg/ml), and the fold decrease due to FK506 was calculated.
[c] WT, wild type.

2 have equivalent mutations but differ in their wild-type versus *fks*Δ strain backgrounds. Mutants WT-C1 and fks2Δ-M1 have Fks1-F625Δ mutations, mutants WT-A2 and fks1Δ-M1 have Fks2-S663T mutations, and multiple mutants, including WT-C2 and fks1Δ-C2, have Fks2-F659Δ mutations. For all three pairs, the mutations had dramatically increased effects in the *fks*Δ backgrounds. Specifically, the Fks1-F625Δ mutation decreased echinocandin susceptibility 8- to 32-fold in the WT background and 128- to 512-fold in the *fks2*Δ backgrounds. Similarly, in the WT and *fks1*Δ backgrounds, the Fks2-F659Δ mutation decreased susceptibility 2- to 4-fold and 64- to 256-fold, respectively, and the Fks2-S663F mutation decreased susceptibility 4- to 8-fold and 128- to 256-fold, respectively. In other words, Fks1-Fks2 redundancy in the WT background attenuated the impact of these mutations 32- to 64-fold (medians for Fks1 and Fks2 mutations, respectively).

***fks1*Δ disruptants are specifically susceptible to the calcineurin inhibitor FK506.** As noted above, *FKS1* in *S. cerevisiae* was first defined by the sensitivity of null mutants in that gene to FK506, a calcineurin inhibitor and widely used immunosuppressive agent (tacrolimus). Similarly, while FK506 (0.3 μg/ml) had no effect on the growth of the *C. glabrata fks2*Δ, *fks3*Δ, and *fks2*Δ *fks3*Δ strains, it inhibited growth (measured spectroscopically in liquid cultures) of the *fks1*Δ and *fks1*Δ *fks3*Δ strains 91% and >99%, respectively, relative to FK506-free controls at 24 h. However, by 48 h, the *fks1*Δ strain fully recovered, while inhibition of the *fks1*Δ *fks3*Δ strain was sustained, implying that Fks3 supported growth to a limited extent. Cultures and isolated colonies obtained following recovery from FK506 inhibition were retested and exhibited the identical patterns of FK506 inhibition (data not shown), indicating that this recovery was not mediated by selection for FK506-resistant mutants.

Fks2-mediated resistance is reversed by FK506. Fks2 mutations outnumber Fks1 mutations 2 to 1 among echinocandin-resistant clinical isolates as noted above, and they also predominate following laboratory selection on echinocandin-containing medium (Table 2). In light of the FK506 susceptibility of *C. glabrata fks1*Δ strains (see above), we reasoned that resistance mediated by an Fks2, but not Fks1, mutation would be reversed by FK506. This was confirmed in susceptibility assays with 4 representative laboratory mutants and, importantly, 6 clinical isolates (Table 3).

FK506 effects are mediated by *C. glabrata* orthologs of Cmp2 and Fpr1. To confirm that FK506 reversal of Fks2-mediated resistance is mediated by calcineurin inhibition, the Cmp2 catalytic subunit gene was disrupted in wild-type 200989 and its C1 (Fks1-F625Δ) and A1 (Fks2-659Δ) derivatives (Table 2). There was no net effect of the *cmp2*Δ deletion on the echinocandin MICs exhibited by 200989 and 200989-C1, i.e., they remained susceptible and resistant, respectively. In contrast, the *cmp2*Δ mutation reversed the echinocandin resistance of 200989-A1 (ANF, CSF, and MCF MICs were reduced 4-, 8-, and 16-fold, respectively).

To inhibit calcineurin, FK506 must bind to the peptidyl-prolyl isomerase Fpr1 (1). Two null mutants of the *C. glabrata* Fpr1 ortholog (isolated by selecting for resistance to the highly synergistic combination of terbinafine plus FK506 and then screening for unaltered terbinafine susceptibility) and their wild-type parent were tested for susceptibility to CSF plus FK506; only the parent demonstrated increased (4-fold) susceptibility in the presence of FK506.

FK506 and *cmp2*Δ effects may be mediated at levels other than *FKS2* expression. In *S. cerevisiae*, *FKS1* is abundantly expressed during normal vegetative growth while *FKS2* and *FKS3* are minimally expressed unless induced by Ca^{2+} or pheromone; this induction is blocked by FK506 or calcineurin mutation (27). These transcriptional effects are presumed to mediate the growth inhibition by FK506 of *fks1* null mutants. In *C. glabrata*, in contrast, *FKS2* is expressed at levels more comparable to those for *FKS1* (*FKS2*/*FKS1* ratios = 0.13 to 3.4), while again, *FKS3* is expressed only minimally (16). Here we extended this analysis to examine the effects on *C. glabrata FKS* expression ratios of brief (30 min) exposure to $CaCl_2$ (30 mM) or CSF (1 μg/ml). Effects of these treatments were compared in the presence and absence of

TABLE 4 *FKS2/FKS1* expression ratios determined by qRT-PCR, and change relative to untreated strain 200989[a]

	Expression ratio and change (Δ) of each strain relative to the untreated strain 200989					
	200989		200989 plus FK506		200989 *cmp2*Δmutant	
Treatment	*fks2/fks1*	Δ	*fks2/fks1*	Δ	*fks2/fks1*	Δ
No treatment	0.17 ± 0.03	1	0.14 ± 0.03	0.8	0.10 ± 0.02	0.6
$CaCl_2$	0.37 ± 0.04	2.2	0.10 ± 0.01	0.6	0.13 ± 0.02	0.8
CSF	0.33 ± 0.15	2.0	0.25 ± 0.04	1.5	0.20 ± 0.01	1.2

[a] Log-phase cultures of wild-type strain 200989 (with or without 1 μg/ml FK506) and the 200989 *cmp2*Δ strain were untreated or treated with $CaCl_2$ (30 mM) or CSF (1 μg/ml) for 30 min, followed by RNA extraction and qRT-PCR analysis of the *FKS2/FKS1* expression ratios (means and standard deviations).

FK506 (1 μg/ml) and in wild-type 200989 versus its *cmp2*Δ disruptant. Under all conditions tested, *FKS3* was expressed at levels >100-fold below those of *FKS1* (not shown). As shown in Table 4, $CaCl_2$ treatment increased the *FKS2/FKS1* ratio a modest 2.2-fold, a result which was blocked by FK506 treatment or *cmp2*Δ disruption. CSF exposure yielded a similarly modest 2.0-fold increase, which was only partially blocked by FK506 or the *cmp2*Δ mutation. Consistent with these results, we detected no effect of FK506 on the growth in SD-ura medium of the *fks2*Δ strain described above in which the *URA3* coding sequence is expressed from the *fks2* promoter (data not shown). These results raise the possibility that calcineurin regulates Fks2 activity in *C. glabrata* at levels other than transcriptional regulation.

DISCUSSION

The genomes of ascomycetous yeasts, including *S. cerevisiae*, *C. albicans*, and *C. glabrata*, encode three Fks paralogs. In *S. cerevisiae*, they have primary roles in vegetative growth (Fks1) and spore wall formation (Fks2 and Fks3) (9, 19, 20, 27). However, the viability, albeit with a growth defect, of *S. cerevisiae fks1*Δ strains indicates that Fks1-Fks2 are partially redundant for vegetative growth. In *C. albicans*, this redundancy appears to be lacking, as *fks1*Δ strains are nonviable (10, 29). In this respect, *C. albicans* is more similar to fungi such as *Aspergillus fumigatus* and *Cryptococcus neoformans*, which encode a single essential Fks1 (17, 43). In contrast, in *C. glabrata*, Fks1 and Fks2 appear to be fully redundant for growth.

We speculate that this true redundancy reflects an evolutionary shift in *C. glabrata* Fks2 function in response to an apparent lack of sporulation in this organism and the potential benefits of Fks1-Fks2 redundancy. Consistent with this shift, resistance-conferring mutations commonly occur in Fks2 of *C. glabrata* clinical isolates (indeed, at twice the apparent frequency of Fks1 mutations), while they occur exclusively in Fks1 of wild-type *S. cerevisiae* and *C. albicans*. This shift is also consistent with the more equivalent levels of *FKS1-FKS2* expression in *C. glabrata* (16) compared to *FKS1*-dominant expression in *S. cerevisiae* (27).

Beyond its evolutionary significance, this shift in *C. glabrata* Fks2 function is likely to have clinical implications. Specifically, Fks1-Fks2 redundancy reduced the rate and impact of resistance-conferring mutations. This was clearly evident in comparisons to *fks1*Δ strains, which generated mutants, all involving Fks2 hot spot 1, at higher frequencies and with the most elevated echinocandin MICs. This was less evident, however, in comparisons to *fks2*Δ strains, suggesting that Fks1 mutations are more likely to confer reduced fitness. Consistent with this, Garcia-Effron et al. (16) showed that hot spot mutations in Fks1 had a more significant effect on glucan synthase V_{max} than mutations in Fks2.

An additional consequence of this redundancy in *C. glabrata* is that the echinocandin resistance level conferred by Fks1 or Fks2 mutation will likely depend on the relative expression of their genes, which in clinical isolates varies more than 20-fold (16). In support of this, the resistance conferred by Fks2 but not Fks1 mutation was reversed to various degrees following treatment with the calcineurin inhibitor FK506 or disruption of its catalytic subunit gene *cmp2*. Calcineurin is specifically required for Fks2 activity in *C. glabrata*, as evidenced by its toxicity to *fks1*Δ (particularly *fks1*Δ *fks3*Δ) strains but not *fks2*Δ strains. FK506 (tacrolimus) is a widely used immunosuppressant in transplant patients; these data imply that *C. glabrata* infections in these patients are less likely to develop echinocandin resistance, especially since this resistance, both clinically and in the lab, is more commonly due to Fks2 than Fks1 mutation. The potential influence on fungal infection of synergistic antifungal-immunosuppressant interaction has been noted (42). Of course, with respect to *C. glabrata* therapy, a more ideal calcineurin inhibitor would be nonimmunosuppressive while retaining the ability to block Fks2-mediated resistance.

In *S. cerevisiae*, the calcineurin requirement of *fks1* null mutants is mediated at the level of *FKS2* expression (27). In *C. glabrata*, our analysis similarly showed that *FKS2* expression can be induced by Ca^{2+} or CSF and that this induction can be blocked to various extents by FK506 treatment or in a *cmp2*Δ strain. However, these effects were very modest compared to those reported for *S. cerevisiae*. This difference no doubt reflects the differences in the level of constitutive *FKS2* expression in these two yeasts. Therefore, to explain the potent effects of FK506 on *C. glabrata*, alternative mechanisms to transcriptional regulation warrant consideration. For example, in *S. cerevisiae*, the mitogen-activated protein (MAP) kinase Smk1 negatively regulates Fks2 activity during spore wall formation, presumably through phosphorylation (18). If the Smk1 ortholog or other kinase has a similar negative effect on *C. glabrata* Fks2, the phosphatase activity of calcineurin may be required to restore its activity.

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